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Immunocytochemical localization of hypocalcin in the endocrine cells of the corpuscles of Stannius in three teleost species (trout, flounder and goldfish)

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Summary. In order to identify the cell-type responsible for the production of hypocalcin (the recently isolated hypocalcemic hormone of teleost fish), the corpuscles of Stannius (CS) of trout, flounder and goldfish, were immunocytochemically stained with antisera raised against trout hypocalcin. The secretory granules of the type-1 cells of the CS, considered to be the hypocalcin-producing cells, showed intense immunoreactivity in all species examined. However, in trout and flounder, the secretory granules produced by the type-2 cells, which have been suggested to represent a functionally different cell-type, also showed an intense immunoreactivity. In goldfish, no type-2 cells were observed. We tentatively conclude that type-1 and type-2 cells represent structurally different forms of the same functional cell-type.

Key words: Corpuscles of Stannius – Hypocalcin – Immunohistochemistry – *Carassius auratus* – *Hippoglossoides elassodon* – *Salmo gairdneri*

Fish are able to regulate their plasma calcium levels effectively. Several hormones have been suggested to be involved in the control of plasma calcium levels: prolactin and cortisol, both of which have hypercalcemic actions in some teleosts (Pang et al. 1973; Wendelaar Bonga and Flik 1982; Flik and Perry 1988), and hypocalcemic factors produced by the ultimobranchial glands and the corpuscles of Stannius (CS) (Fenwick 1982). The CS are small endocrine glands associated with the kidneys and are found exclusively in holostean and teleostean fish (Pang 1973; Wendelaar Bonga and Pang 1986). The presence of a hypocalcemic factor in these glands was originally demonstrated by Fontaine (1964), who observed severe hypercalcemia after removal of the CS in European eels. This hypercalcemia can be reduced by injection of homogenates of the corpuscles.

Recently, the hypocalcemic factor of coho salmon (Wagner et al. 1986), and rainbow trout (Lafeber et al. 1988a, b) has been isolated. It is a glycoprotein, called teleocalcin or hypocalcin, which appears under reducing conditions as a 28000–32000 daltons product. Both substances inhibit Ca^{2+} influx in trout (Wagner et al. 1986; Lafeber et al. 1988b).

Trout hypocalcin, the name first proposed for the hypocalcemic factor of the CS (Pang et al. 1974) and therefore preferred in this paper, was shown to reduce the high plasma calcium levels in European eels from which the CS had been removed (Hanssen et al. 1988). The N-terminal amino acid sequence of the hypocalcemic factors of salmon and trout (Wagner et al. 1986; Lafeber et al. 1988a) showed great similarity to the predicted sequence of the CS product of Australian eel (Butkus et al. 1987).

A question still open for debate concerns the cellular origin of hypocalcin. In a variety of fish, the presence of two endocrine cell-types has been suggested (for review, see Wendelaar Bonga and Pang 1986). One cell-type (type-1) is more abundant and generally ovoid. It contains an extensive granular endoplasmic reticulum and is further characterized by the presence of numerous large secretory granules. The other cell-type (type-2) has slender irregular cell bodies. The granular endoplasmic reticulum is limited and only a few small secretory granules are usually found.

The occurrence of two structurally different cell-types has been tentatively related to the aquatic environment of the fish: in reviewing the pertinent literature, Wendelaar Bonga and Pang (1986) have concluded that all stenohaline marine fish possess only type-1 cells. The CS of freshwater fish or euryhaline fish spending part of their life cycle in freshwater have been reported to contain both cell-types (Wendelaar Bonga and Pang 1986). From this divergence, it seems plausible to conclude that the two cell-types have different functions and produce different hormones. Type-1 cells are thought to produce the hypocalcemic principle (Wendelaar Bonga et al. 1980; Urasa and Wendelaar Bonga 1985; 1987; Kaneko et al. 1988). They become activated upon transfer of euryhaline fish from fresh water to seawater (Cohen et al. 1975; Wendelaar Bonga et al. 1976; Meats et al. 1978). This activation is related to the change in ambient Ca^{2+} (Wendelaar Bonga et al. 1976; 1980; Meats et al. 1978), as it does not occur when fish are transferred from fresh water to calcium-deficient seawater (Cohen et al. 1975; Wendelaar Bonga et al. 1980). This cell-type is also activated in high-calcium fresh water or during ovarian maturation, conditions associated with hypercalcemia (Urasa and Wendelaar Bonga 1985, 1987). Conversely, type-2 cells are not affected by changes in the calcium content of the ambient water (Wendelaar Bonga et al. 1976, 1980; Meats et al. 1978), and their activity is reduced by fresh water to seawater transfer (Meats et al. 1978; Wendelaar Bonga et al. 1976; 1980).

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Although ultrastructural evidence may be advanced for the presence of two cell-types in the CS, conclusive physiological evidence that the structurally different cell-types are also functionally different has not been presented so far. Some authors have questioned the functional distinction of the two cell-types. Lopez and coworkers (1984) tentatively conclude on the basis of light-microscopical observations that both cell-types of the eel CS degranulate after experimentally induced hypercalcemia, and they suggest that the cell-types represent structurally different stages of one functional endocrine cell-type. A recent electron-microscope study in our laboratory (Lafeber and Perry 1988) indeed shows that the degree of degranulation of type-1 and type-2 cells is similar after intra-peritoneal CaCl_2 -injection. Yet, a non-specific effect of Ca^{2+} on hormone-release by the two cell-types cannot be excluded. We have taken the ultrastructural immunocytochemical localization of the hypocalcin antigen as an indication of the presence of hypocalcin-producing cells, and have studied the CS of fish species containing either type-1 or type-1 and type-2 cells.

Materials and methods

Animals

Freshwater rainbow trout (*Salmo gairdneri*) were obtained from a trout hatchery, and goldfish (*Carassius auratus*) from a commercial dealer. These fish were kept in the laboratory in tanks with running tap water for at least three weeks. The water contained 1.0 mM Ca^{2+} . Seawater flounder (*Hippoglossoides elassodon*) were netted in the Pacific Ocean near Bamfield, British Columbia. The CS were collected and fixed immediately after capture of the fish. The weight of the fish was about 170 g, 50 g and 450 g, respectively, for the three species. The fish used were of both sexes and their gonads were immature.

Electron microscopy and immunogold labeling

The fish were quickly killed by an overdose of tricaine methanesulphate (MS 222; 2 g/l) and the CS were removed. For examination of the general ultrastructure CS were prefixed in glutaraldehyde and fixed in a mixture of glutaraldehyde, potassium dichromate and osmium tetroxide (Wendelaar Bonga et al. 1980). For immunogold labeling, CS were fixed by immersion in a solution of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.5 for 2 h. After a 30-min wash in 0.1 M sodium phosphate buffer pH 7.5, some of the tissues were dehydrated in serial ethanol. The tissues were embedded in Spurr's resin. Ultrathin sections (90–140 nm) were cut using glass knives and mounted on nickel grids. Protein-A-gold (PAG) labeling was carried out according to the method of Roth et al. (1978) with some modifications (Kaneko et al. 1985). Grids were first placed on drops of sodium phosphate-buffered saline (PBS) containing 1% ovalbumin for 10 min to reduce non-specific binding, and were next incubated with one of two trout hypocalcin antisera raised in rabbits (RADH I and II; Kaneko et al. 1988) at dilutions of 1:250, 1:1000 and 1:4000 for 2 h. A dilution of 1:1000 was considered most appropriate. After six washes of 5 min each with PBS, the grids were placed on drops of PAG, diluted 1:50 with PBS, for 1 h, and washed with PBS and then with distilled water. All steps were conducted at room temperature. The sections were poststained with uranyl acetate

(5 min) and lead citrate (1 min), and examined using a Philips 301 electron microscope. The specificity of the immunocytochemical staining was confirmed by control procedures including the substitution of the specific antiserum by normal serum and preabsorption of the antiserum with trout hypocalcin (at least 10 $\mu\text{g}/\text{ml}$) at the lowest working dilution). The antisera used have been characterized extensively. The specificity was determined by immunodiffusion, RIA, ELISA, and immunocytochemistry. No cross reactivity was found with several vertebrate hormones, including PTH, FSH, LH, TSH, CT, angiotensin I and II, and somatostatin (Kaneko et al. 1988).

Results

Two ultrastructurally different types of granule-containing cells are observed in the CS of two of the three species examined; type-1 cells, easily recognized by their numerous large granules, and type-2 cells, which contain few small granules (Fig. 1). According to a conservative estimate, type-1 and type-2 cells in rainbow trout and in flounder represent 75% and 25%, respectively, of the granule-containing cells in the CS; in the goldfish CS, type-2 cells could not be found.

Rainbow trout

The ultrastructure of the cells is similar to the description given by Meats et al. (1978) for this species. The cell bodies of the type-2 cells differ in shape, which varies from ovoid to slender and stellate (Fig. 1). Diameters of type-1 cell granules vary between 400 and 800 nm, and of the type-2 cell granules between 150 and 350 nm.

Flounder

Type-1 cells are ovoid, contain numerous, large secretory granules (diameter 250–400 nm), and show moderately electron-dense cytoplasm. Type-2 cells are stellate, with long slender processes lying between the type-1 cells, and show an electron-translucent cytoplasm. In the latter cell-type, the incidence of secretory granules is lower and their diameter significantly smaller (100–200 nm) than in type-1 cells.

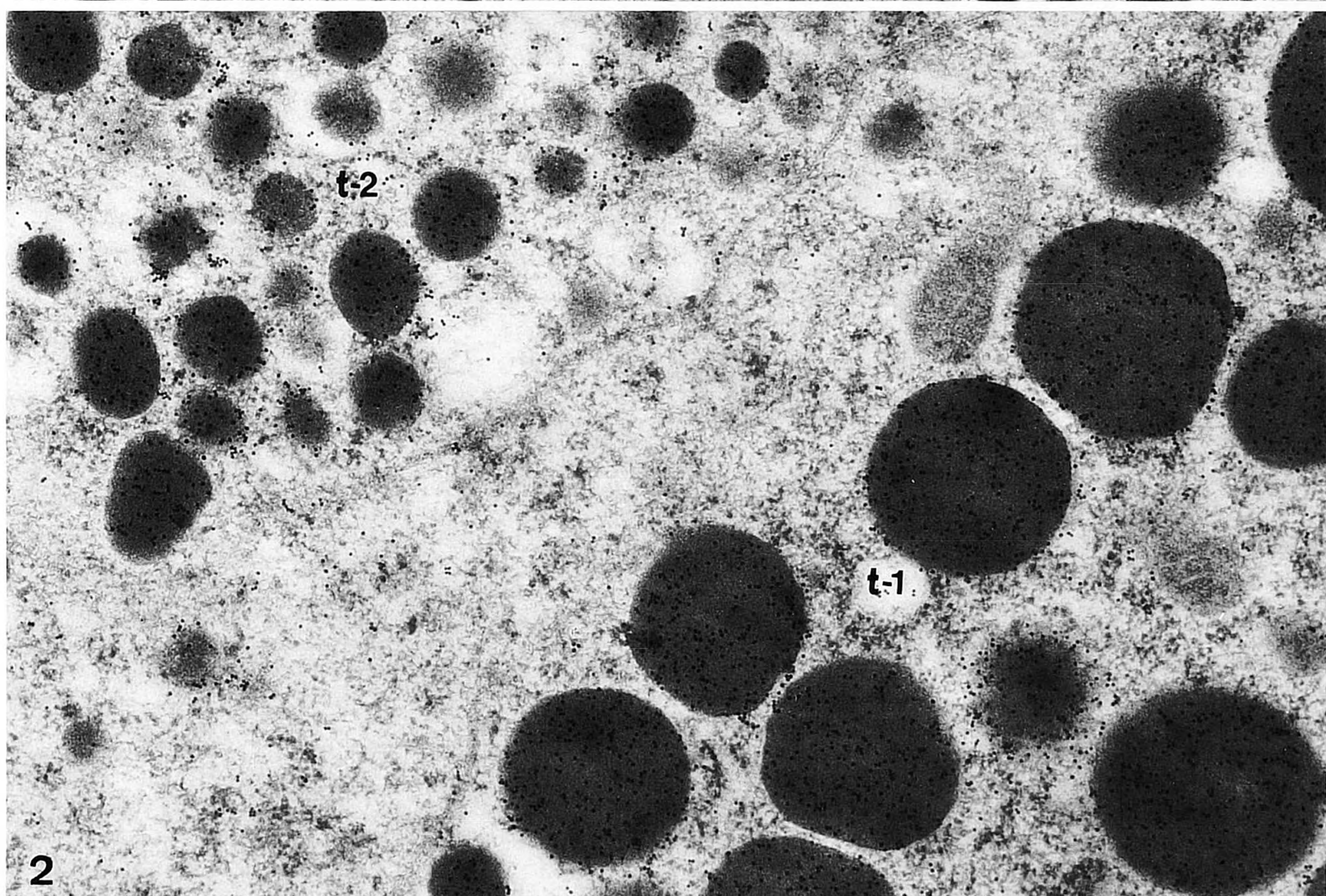
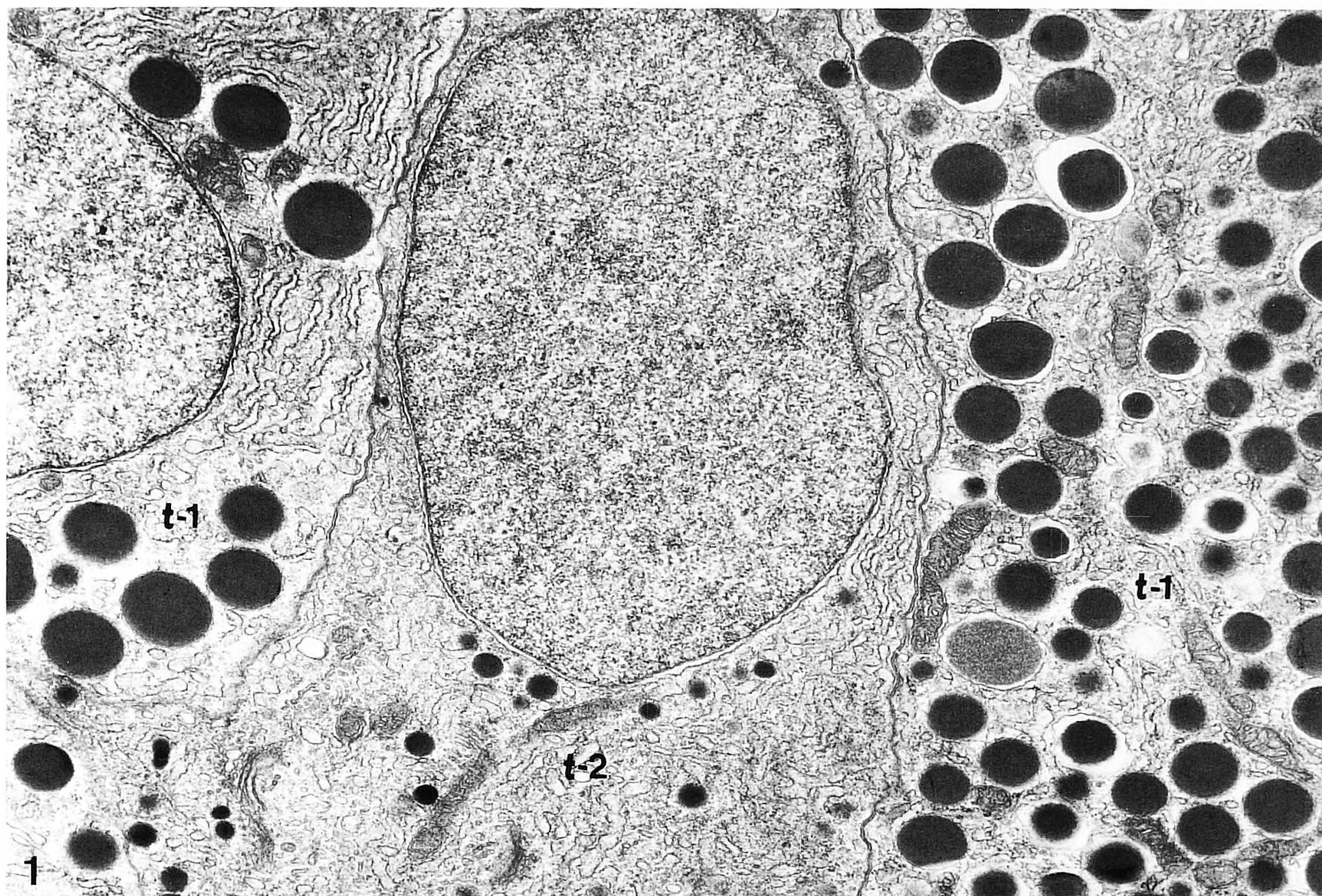
Goldfish

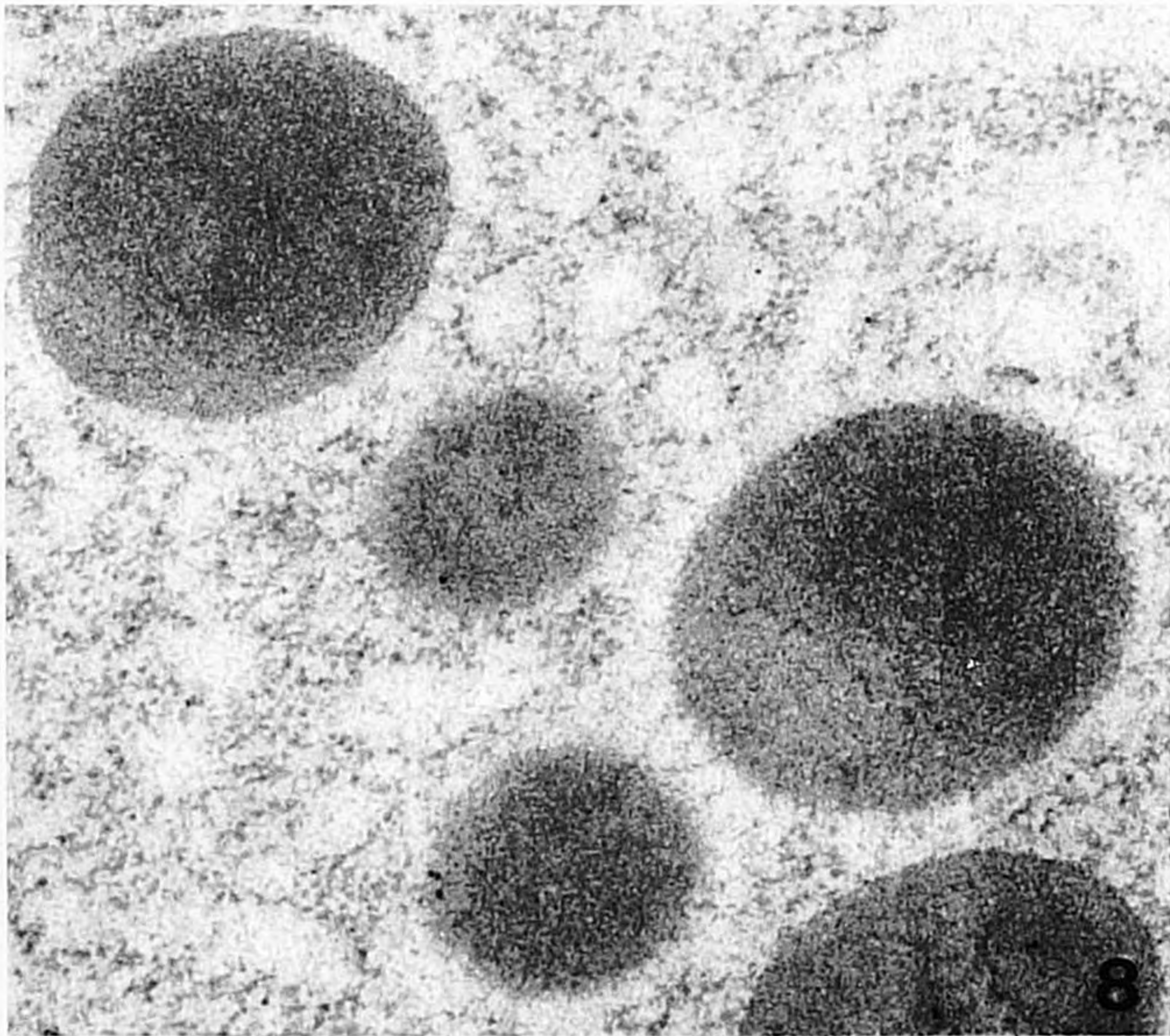
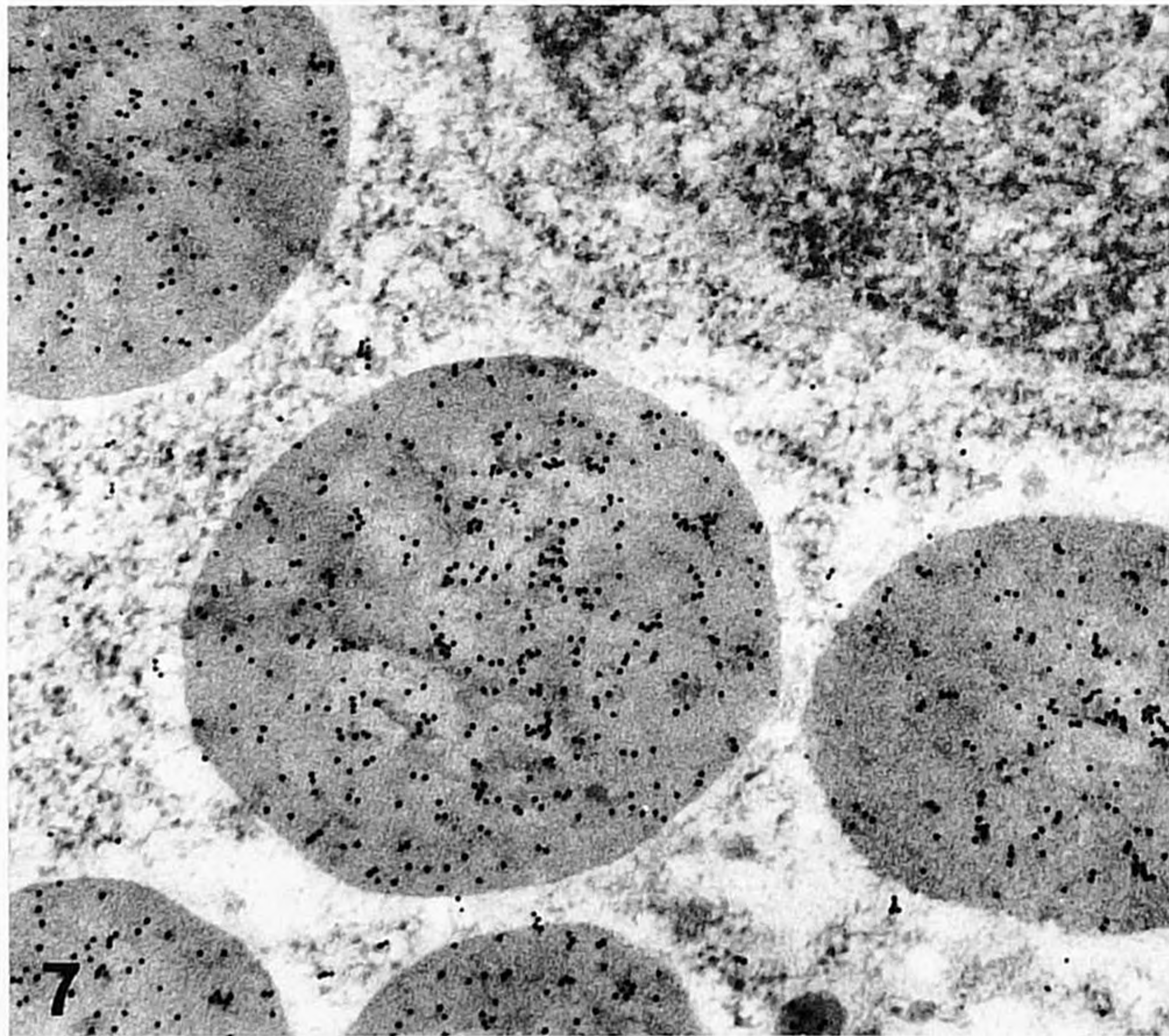
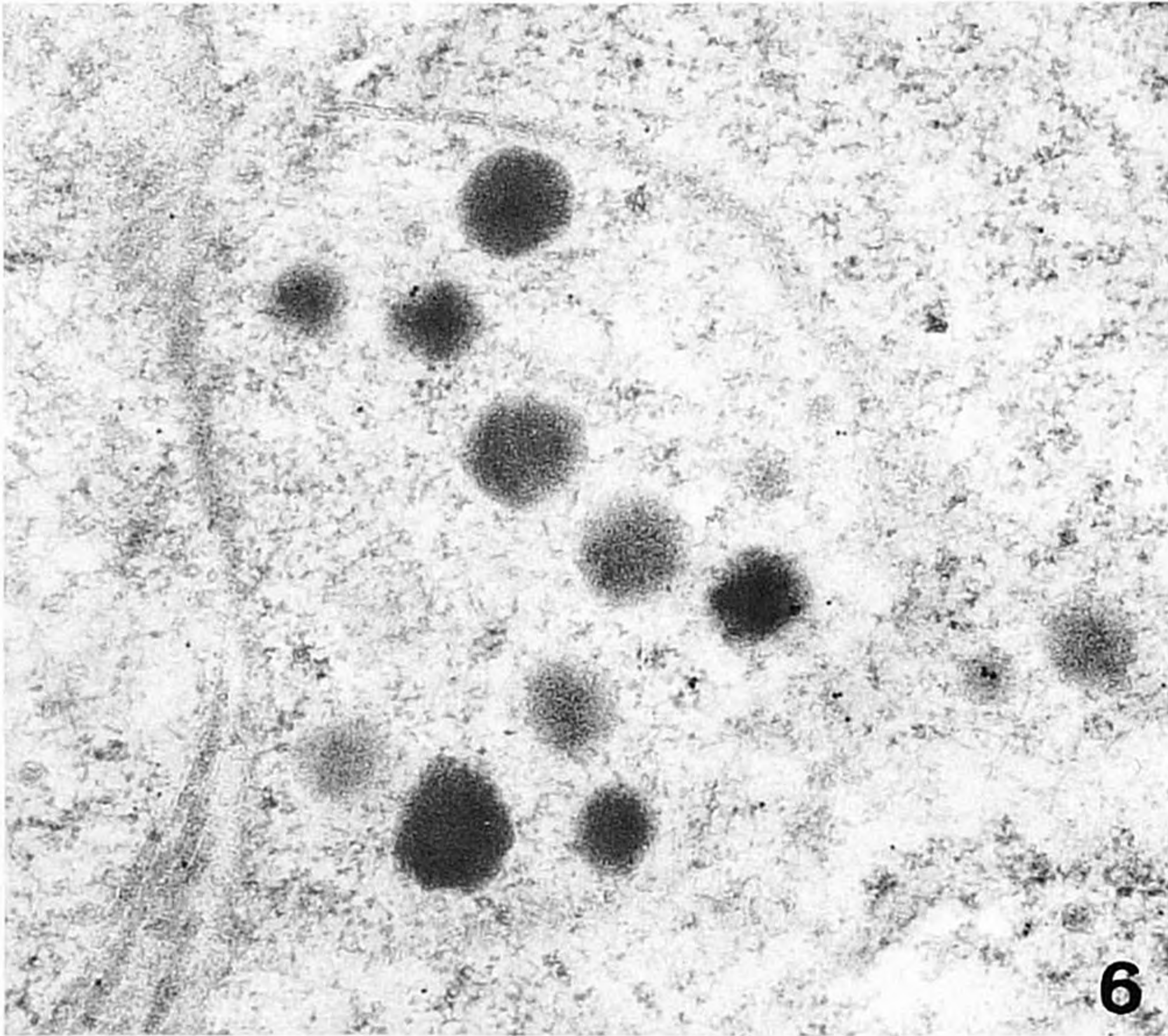
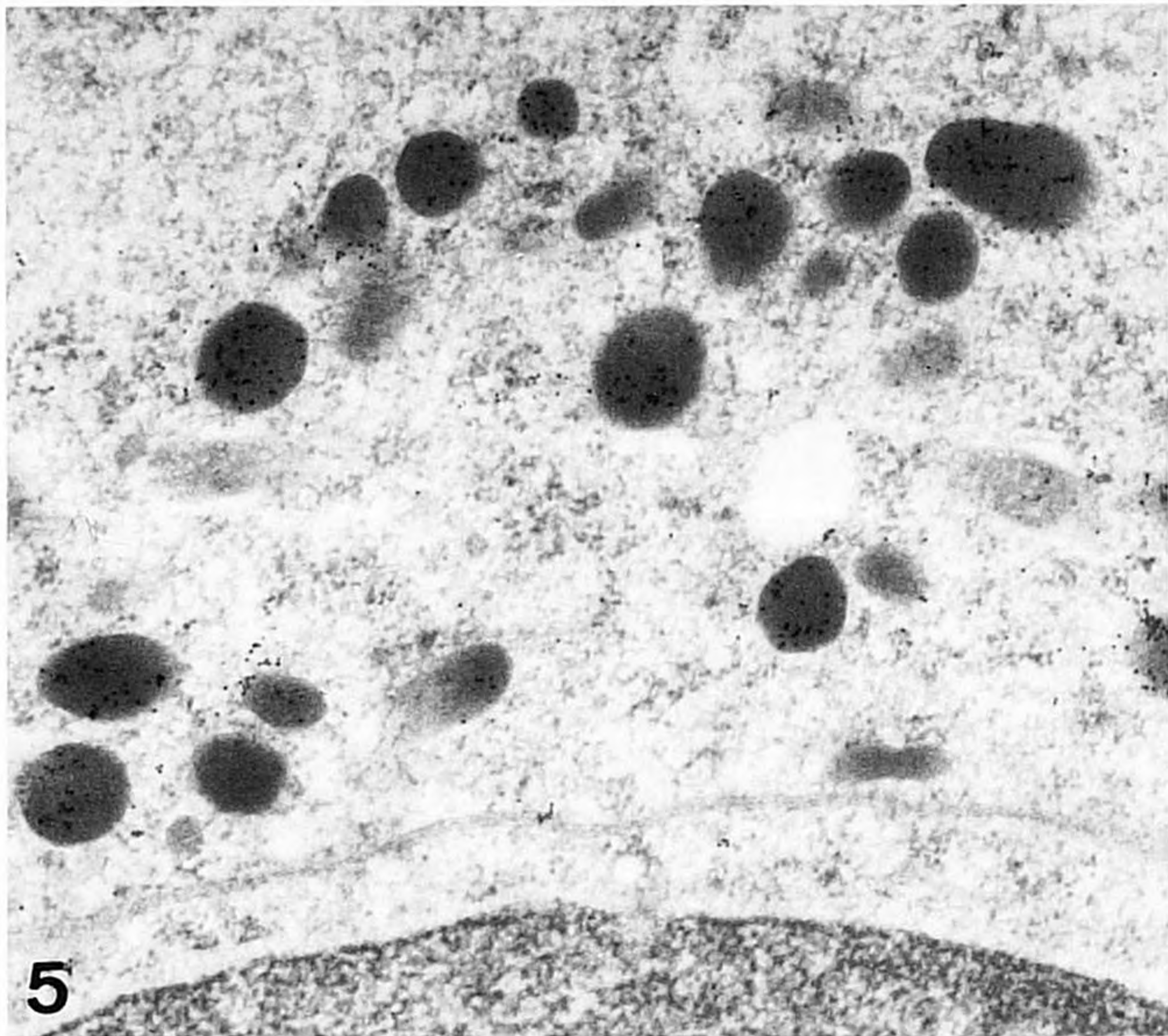
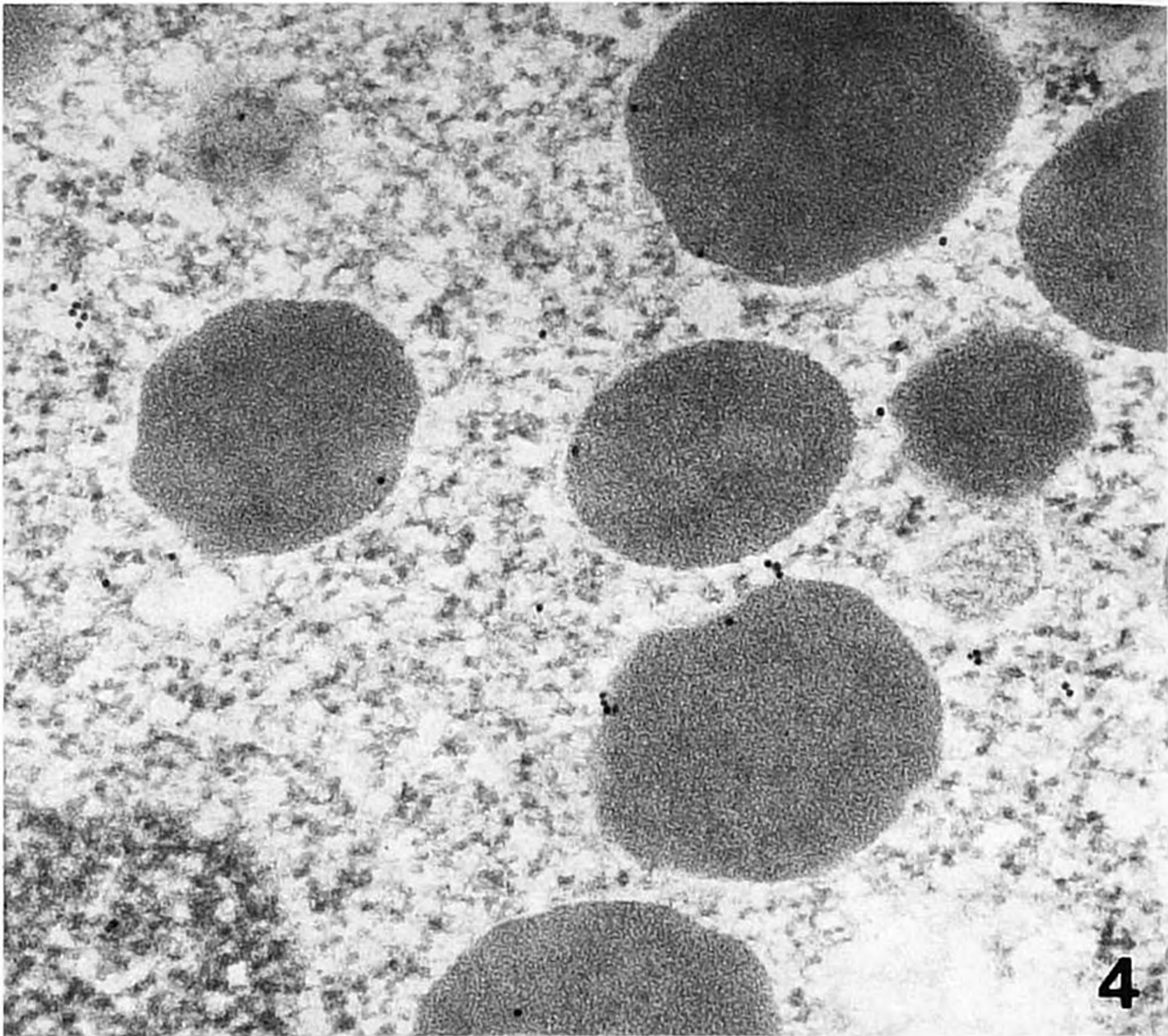
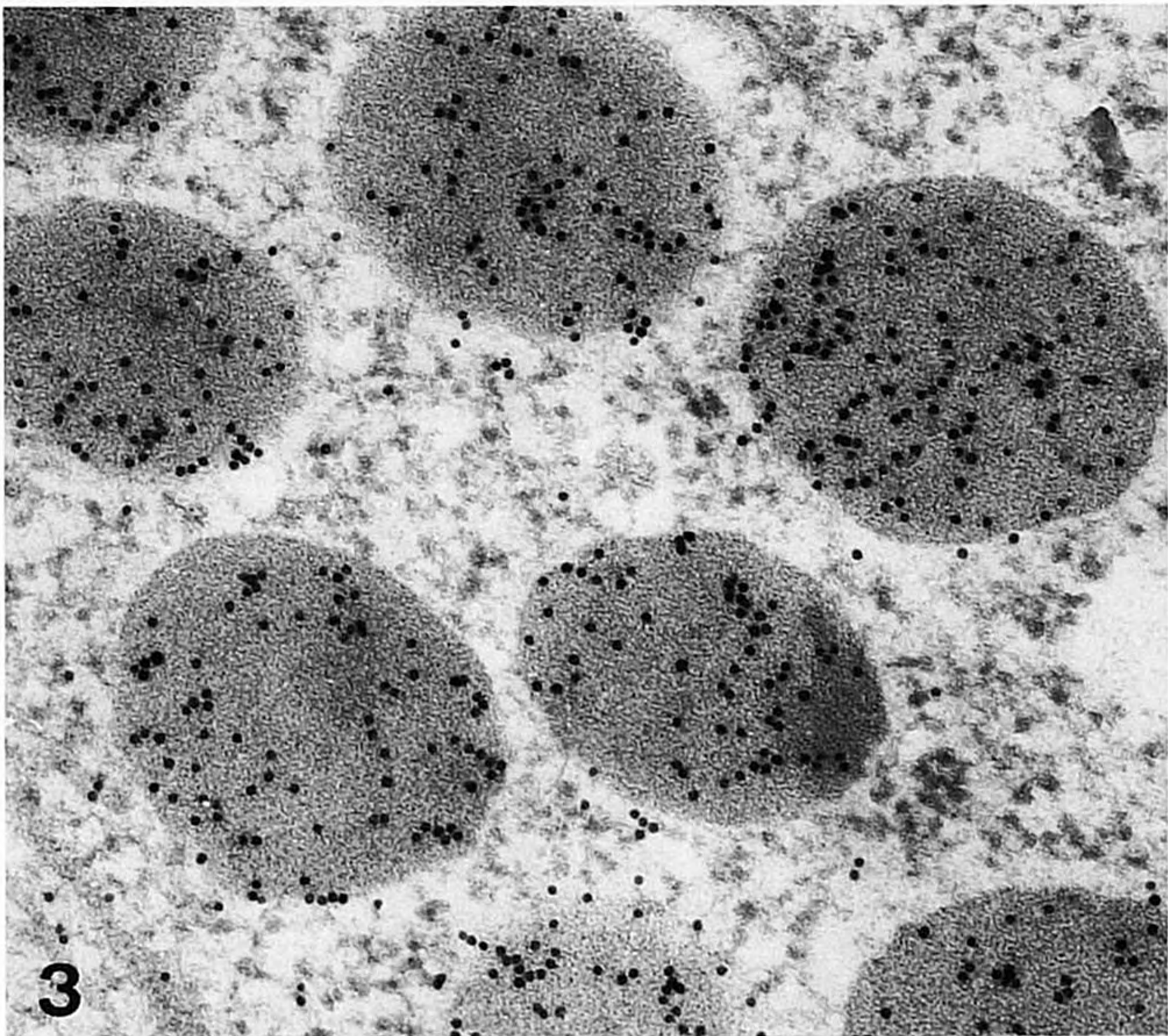
Our observations on the type-1 cells of goldfish confirm our previous description of these cells (Wendelaar Bonga et al. 1980). An important difference from the observations on the goldfish CS made in our earlier study is the absence of type-2 cells. Type-1 cells are ovoid and contain large granules (500–950 nm).

Fig. 1. Corpuseles of Stannius (CS) of trout. Type-1 cells (*t-1*), with large secretory granules, and type-2 cells (*t-2*) with small secretory granules. Glutaraldehyde-potassium dichromate-osmium tetroxide fixation, and uranyl acetate and lead citrate staining. $\times 18000$

Figs. 2–8. Cells of CS fixed with paraformaldehyde and glutaraldehyde, and stained with uranyl acetate and lead citrate. **Figs. 2, 3, 5 and 7** were treated with anti-trout hypocalcin antiserum (dilution 1:1000), followed by immunogold labeling

Fig. 2. CS of trout, showing secretory granules of type-1 cells (*t-1*) and type-2 (*t-2*) cells, stained positively for hypocalcin. $\times 50000$





Immunogold labeling

In trout, both type-1 and type-2 cells show immunoreactivity to both hypocalcin antisera (Fig. 2). The immunogold label is confined almost exclusively to the secretory granules. No specific staining of the Golgi areas and the granular endoplasmic reticulum is observed. Almost all type-1 secretory granules show a clear and homogeneous staining reaction. Preabsorption of the antiserum with trout hypocalcin results in a complete loss of specific immunogold labeling (not shown). In the CS of flounder the distribution of the label is similar to that in trout CS: in both cell-types, the granules are labeled (Figs. 3, 5). The type-1 cells of goldfish also show considerable labeling (Fig. 7). The intensity of labeling is slightly less intense than that observed in the CS of trout. The preabsorption controls are negative (Figs. 4, 6, 8).

Discussion

Although the ultrastructure of the type-1 and type-2 cells of the CS in the three species investigated is remarkably different, the granules of both cell-types react to antisera raised against hypocalcin, the hormone we have isolated from trout CS. This glycoprotein inhibits the branchial influx of calcium from the water in trout and decreases the hypercalcemia of stanniectomized eels (Lafeber 1988; Hansen et al. 1988); it therefore probably represents the hypocalcemic hormone produced by the CS. The antisera used in this study have been extensively analyzed and their high specificity demonstrated (Kaneko et al. 1988).

The presence of immunoreactivity in the granules of both cell-types may indicate that these cells produce the same product and therefore represent different stages of one functional type of endocrine cell. This conclusion contrasts with reports by us (Wendelaar Bonga et al. 1976, 1980) and others (Meats et al. 1978); these reports have suggested the presence of two functional cell-types in the CS. With the light microscope, two cell-types have been described in the CS of teleosts on the basis of the presence or absence of staining affinity for conventional stains, in particular periodic-acid-Schiff (PAS), paraldehyde fuchsin, and Bowie's stain (Krishnamurthy and Bern 1969; Krishnamurthy 1976). Krishnamurthy and Bern (1969) have demonstrated that the PAS-positive and PAS-negative cells in the CS of trout are ultrastructurally distinct, and show the characteristics of the cells that we have called type-1 and type-2 cells in sticklebacks, eels (Wendelaar Bonga and Greven 1975; Wendelaar Bonga et al. 1976), goldfish, and killifish (Wendelaar Bonga et al. 1980). Similar cells have been described in, e.g., trout (Meats et al. 1978), coho salmon

(Aida et al. 1980), tilapia (Urasa and Wendelaar Bonga 1985), and the holostean *Lepisosteus platyrhynchus* (Bhattacharya et al. 1982). In almost all of these species, the type-1 and type-2 cells show similar marked differences in cell-shape, granule-size and extent of the granular endoplasmic reticulum.

All the above-mentioned species are typical of freshwater fish or euryhaline fish spending part of their life cycle in freshwater. In the CS of the few seawater fish studied so far, only the presence of cells with the characteristics of type-1 cells has been reported: cod, plaice (Wendelaar Bonga and Greven 1975) and toadfish (Bhattacharya and Butler 1978).

In euryhaline fish, the type-1 cells are more active in seawater than in fresh water (Wendelaar Bonga et al. 1976, 1980; Meats et al. 1978). The reverse has been reported for type-2 cells in sticklebacks, trout, killifish (Wendelaar Bonga et al. 1976, 1980), eels (Olivereau and Olivereau 1978), and coho salmon (Aida et al. 1980). The differences between type-1 and type-2 cells in staining characteristics, ultrastructure and response to fresh water or seawater have been interpreted as indications that the two cell-types are functionally different. The present results do not support this conclusion and point to the production by both cell-types of the same hormone. The possibility that the structural diversity of the CS cells reflects different stages of one functional cell-type rather than two different kinds of cells has been raised earlier. Recently, Lopez et al. (1984) have come to this conclusion on the basis of the light microscopical observation that all CS cells of eels show immunoreactivity to an antiserum raised against PTH. However, eels usually have only few type-2 cells (personal observation), and most of the few small granules of the type-2 cells are probably beyond detection with the light-microscope. This is illustrated by our earlier light-microscope study on the CS of goldfish (Kaneko et al. 1988). With the same antiserum as used in the present study, reactivity to antihypocalcin is only seen in type-1 cells, in contrast to the present ultrastructural observations.

There are some other indications that the type-2 cells, albeit ultrastructurally very distinct, may not represent a functionally separate cell-type. Firstly, Wendelaar Bonga et al. (1980) have found that, in the CS of goldfish, the type-2 cells are prominent and make up 20 to 30% of the total number of gland cells. In the goldfish examined in the present study, type-2 cells are absent. Secondly, we have recently found that type-2 cells do not occur in the CS of the freshwater catfish *Heteropneustes fossilis*, whereas they are common in the stenohaline seahorse and flounder, both from seawater (Ahmad and Wendelaar Bonga, unpublished). Thus, our earlier conclusion that type-2 cells are typical of stenohaline or euryhaline freshwater fish seems no longer tenable. Thirdly, Lafeber and Perry (1988) have recently shown that both type-1 and type-2 cells release large numbers of secretory granules following artificially induced severe hypercalcemia. This response is not observed in pituitary cells and has therefore been interpreted as a specific response resulting in the release of a hypocalcemic hormone. It may seem remarkable that structurally distinct cell-types produce the same hormone. However, although exceptional, it is not unique. For instance, in the rat pituitary gland, Van Putten and Kiliaan (1988) have distinguished four ultrastructurally different cell-types, all of which stain immunocytochemically with antiprolactin. In addition, cells with small round granules, instead of the large polymorphic granules considered to be characteristic

Fig. 3. Secretory granules of flounder type-1 cells, stained positively for hypocalcin. $\times 80000$

Fig. 4. As Fig. 3; preabsorption control. $\times 67000$

Fig. 5. Secretory granules of flounder type-2 cells, stained positively for hypocalcin. $\times 40000$

Fig. 6. As Fig. 5; preabsorption control. $\times 40000$

Fig. 7. Secretory granules of goldfish type-1 cells, stained positively for hypocalcin. $\times 45000$

Fig. 8. As Fig. 7; preabsorption control. $\times 45000$

for prolactin cells, appear to contain prolactin. These observations are in line with the concept of Yoshimura (1986), who has proposed an "entire range" of subtypes of TSH cells and of LH/FSH cells in the male rat; he suggests that each subtype might reflect a different stage of cell differentiation or a different functional phase of one and the same cell.

The hypocalcin antisera do not show species-specificity. From sequencing studies, it is known that the N-terminals of the CS hormone of salmon, Australian eel and trout, are similar (Wagner et al. 1986; Butkus et al. 1987; Lafeber et al. 1988a). The slightly higher density of the gold labeling observed in trout may be related to the fact that the hypocalcin used for raising the antisera was isolated from trout; however the possibility that the secretory granules in trout contain a higher concentration of the hormone cannot be excluded.

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